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# FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2

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**Fibroblast growth factor (FGF) activates a protein kinase cascade in SK-N-MC cells that regulates gene expression at a cyclic-AMP response element (CRE) by stimulating the transcriptional activity of CREB. The activation of CREB is prevented by a dominant negative mutant of Ras and triggered via the same site (Ser133) that becomes phosphorylated in response to cyclic AMP and $Ca^{2+}$. However, the effect of FGF is not mediated by cyclic AMP-dependent protein kinase, TPA-sensitive isoforms of protein kinase-C, $p70^{S6K}$ or $p90^{rsk}$ (all of which phosphorylate CREB at Ser133 *in vitro*). Instead, we identify the FGF-stimulated CREB kinase as MAP kinase-activated protein (MAPKAP) kinase-2, an enzyme that lies immediately downstream of p38 MAP kinase, in a pathway that is also stimulated by cellular stresses. We show that MAPKAP kinase-2 phosphorylates CREB at Ser133 *in vitro*, that the FGF- or stress-induced activation of MAPKAP kinase-2 and phosphorylation of CREB and ATF-1 are prevented by similar concentrations of the specific p38 MAP kinase inhibitor SB 203580, and that MAPKAP kinase-2 is the only detectable SB 203580-sensitive CREB kinase in SK-N-MC cell extracts. We also show that transfection of RK/p38 MAP kinase in SK-N-MC cells, but not transfection of p44 MAP kinase, activates Gal4–CREB-dependent transcription via Ser133. These findings identify a new growth factor and stress-activated signaling pathway that regulates gene expression at the CRE.**

*Keywords*: CREB transcription/FGF/kinase cascade/signaling pathway/stress activation

## Introduction

Fibroblast growth factor (FGF) is a potent mitogenic factor that is also known to initiate changes important for neural differentiation, survival and plasticity (Lo, 1995). Mechanisms underlying these diverse actions of FGF are not understood but may result, at least in part, from the activation of distinct signaling pathways controlling gene expression. Stimulation of FGF receptors is known to activate Ras and a kinase cascade culminating in the activation of p42 and p44 mitogen-activated protein (MAP) kinases (Thomas *et al.*, 1992; Wood *et al.*, 1992). The activated MAP kinases then phosphorylate many key regulatory proteins, including the protein kinase $p90^{rsk}$ (Sturgill *et al.*, 1988) and transcription factors such as Elk-1 (Marais *et al.*, 1993). FGF is also able to activate the MAP kinase homologs SAPK and p38 MAP kinase. SAPK (Kyriakis *et al.*, 1994) is also referred to as JNK (Derijard *et al.*, 1994), and p38 MAP kinase is also called Reactivating Kinase (RK) or CSBP (Han *et al.*, 1994; Lee *et al.*, 1994a; Rouse *et al.*, 1994). Inflammatory cytokines and a variety of different forms of cellular stress activate SAPK, which in turn phosphorylates and activates several transcription factors that may include c-Jun (Binetruy *et al.*, 1991; Pulverer *et al.*, 1991), ATF2 (Gupta *et al.*, 1995), and Elk-1 (Cavigelli *et al.*, 1995; Whitmarsh *et al.*, 1995). Similar stimuli activate p38 MAP kinase (Rouse *et al.*, 1994; Raingeaud *et al.*, 1995) which can activate a downstream kinase called MAPKAP (MAP kinase-activated protein) kinase-2 (Stokoe *et al.*, 1992; Cuenda *et al.*, 1995). The identification of a specific, cell permeable inhibitor of the p38 MAP kinase (Lee *et al.*, 1994a; Cuenda *et al.*, 1995) has recently implicated the p38 MAP kinase signaling pathway in the production of many cytokines, such as IL-1, IL-6, IL-8 and TNFα (Lee *et al.*, 1994b; Beyaert *et al.*, 1996) in the induction of the transcription factors c-Fos and c-Jun (Hazzalin *et al.*, 1996), and in the stimulation of glucose transport (Gould *et al.*, 1995).

To identify signaling pathways transmitting the FGF signal to the nucleus we have studied FGF regulation of the opoid precursor, proenkephalin, in the human neuroblastoma SK-N-MC cell line (Chu *et al.*, 1994; Tan *et al.*, 1994). In these cells FGF activates proenkephalin transcription via a Ras-dependent pathway (Tan *et al.*, 1994) that ultimately converges upon a CRE-like DNA regulatory element (CRE-2), a CREB/ATF binding site shown to mediate gene activation by cAMP- and $Ca^{2+}$/depolarization-dependent pathways (Comb *et al.*, 1986; Van Nguyen *et al.*, 1990). To characterize FGF-dependent signaling at the CRE-2 element we therefore examined the role of CREB, previously implicated in the regulation of neuropeptide and immediate early gene expression (Gonzalez and Montminy, 1989; Lee *et al.*, 1990; Sheng *et al.*, 1991; Ginty *et al.*, 1994). Phosphorylation of CREB at Ser133 regulates its ability to activate transcription in response to cAMP (Gonzalez and Montminy, 1989), increased intracellular $Ca^{2+}$ (Sheng *et al.*, 1991) and NGF (Ginty *et al.*, 1994).

In this paper we demonstrate that FGF stimulates CREB-dependent transcription through an intracellular signaling pathway that requires Ras but does not involve components of the classical MAP kinase pathway. Instead, FGF or chemical stress triggers activation of a pathway

involving p38 MAP kinase and MAPKAP kinase-2. In particular, we show that the phosphorylation of CREB at Ser133 is induced by FGF or chemical stress, and is prevented by the specific p38 MAP kinase inhibitor at concentrations similar to those that inhibit the activation of MAPKAP kinase-2. Moreover, MAPKAP kinase-2 phosphorylates CREB at Ser133 *in vitro*, and transfection of SK-N-MC cells with p38 MAP kinase stimulates transcription of a CREB-dependent reporter gene. These results indicate that FGF and stress-dependent CREB phosphorylation is catalyzed by MAPKAP kinase-2 or a closely related homolog *in vivo*, and hence identify a new signaling cascade that controls CREB phosphorylation and gene expression at the CRE.

## Results

### *CREB activates transcription in response to FGF*

Previous studies have shown that FGF stimulates proenkephalin transcription via a cyclic-AMP response element (CRE-2) (Tan *et al.*, 1994). As CREB is one of many CRE-2 binding proteins present in SK-N-MC cells (Tan *et al.*, 1994), we asked whether CREB might serve to regulate proenkephalin transcription in response to FGF. To eliminate complications arising from endogenous factors interacting at the proenkephalin CRE, we tested the ability of a Gal4–CREB fusion protein (see Figure 1A) to stimulate transcription of a proenkephalin–chloramphenicol acetyltransferase (CAT) reporter gene, pG5-ENKΔ80, where the CRE element was replaced with five copies of a Gal4 binding site (Figure 1A). In a fashion similar to the effects of FGF and cAMP on the wild-type proenkephalin promoter (pENKAT-12), Gal4–CREB activated transcription in response to either FGF or cAMP and treatment with FGF and cAMP together produced a synergistic increase in CAT expression (Figure 1B). FGF-dependent induction of proenkephalin–CAT by Gal4–CREB was dependent upon the CREB part of the Gal4 fusion protein as neither Gal4 alone nor a Gal4–VP16 fusion protein were able to stimulate transcription in response to FGF (see Figure 1B). The strikingly similar profile of FGF and cAMP on Gal4–CREB-dependent transcription and CRE-dependent transcription (pENKAT-12) suggests that CREB may play an important role in transducing the effects of FGF and the FGF/cAMP synergy observed in SK-N-MC cells.

Forskolin and protein kinase A are well known to stimulate the phosphorylation of CREB at Ser133, a site essential for the transcriptional activity of CREB (Gonzalez and Montminy, 1989; Lee *et al.*, 1990; Sheng *et al.*, 1991). Therefore, we next investigated the role of Ser133 in transcriptional activation by FGF. As shown in Figure 1B, mutation of Ser133 largely blocked the ability of CREB to respond to either forskolin or FGF, suggesting that phosphorylation at Ser133 is necessary for FGF to stimulate CREB's transcriptional activity.

### *FGF stimulates CREB phosphorylation at Ser133 in SK-N-MC cells and primary astrocytes*

To determine whether FGF stimulates CREB phosphorylation at Ser133, we analyzed phosphorylation at this site using an antibody specific for the Ser133-phosphorylated form of CREB (see Materials and methods) (Ginty *et al.*, 1993; Hagiwara *et al.*, 1993). As shown in Figure 2A, little if any phospho-CREB is detected in control SK-N-MC cells. However, treatment with 50 ng/ml FGF increased CREB phosphorylation at Ser133 within 5 min, with peak phosphorylation occurring between 15 and 30 min. The antibody used to detect CREB phosphorylated at Ser133 also detects FGF-induced phosphorylation of a 38 kDa protein (lower band in Figure 2A) that co-migrates with ATF-1, a related transcription factor that shares sequence identity surrounding Ser133 (Liu *et al.*, 1993) and cross-reacts with the antibody used (see Materials and methods). No change in CREB or ATF-1 immunoreactivity was detected when the same whole-cell extracts were subjected to immunoblot analysis using antibodies that recognize CREB or ATF-1, regardless of their phosphorylation status at Ser133 (CREB) or Ser63 (ATF-1). As FGF and forskolin act synergistically to stimulate proenkephalin transcription, we next analyzed the effect on CREB phosphorylation of treating SK-N-MC cells with FGF and forskolin. FGF and forskolin together result in an enhancement of CREB/ATF-1 phosphorylation that is roughly equal to the sum of the two individual effects (Figure 2B). This effect was observed at different doses of forskolin and FGF and at different times post-treatment (data not shown).

FGF (Woodward *et al.*, 1992) and FGF receptors (Wanaka *et al.*, 1990) are broadly expressed in the central nervous system by both neurons and glia. As FGF is known to stimulate proenkephalin gene expression in type I astrocytes (Tan *et al.*, 1994), we next asked whether FGF regulates CREB phosphorylation in primary cultures of type I astrocytes prepared from cerebral cortex. Treatment of astrocytes with FGF rapidly induced nuclear CREB phosphorylation as detected immunohistochemically using anti-phospho-CREB antiserum (Figure 2C). The kinetics of nuclear phospho-CREB immunoreactivity were similar to that observed in SK-N-MC cells.

### *FGF-dependent CREB phosphorylation does not require protein kinase A, phospholipase C, TPA-activated PKC isoforms, or p70$^{S6K}$*

We next characterized FGF-activated signaling pathways required to stimulate Gal4–CREB-dependent transcription. As shown in Figure 3A, inhibition of the cAMP pathway by overexpression of a mutant protein kinase A regulatory subunit (RI) (Clegg *et al.*, 1987) blocked forskolin-stimulated CREB transcription, but had no effect on FGF-stimulated transcription. L6 myoblasts stably transfected with a wild-type FGF receptor (L6-FGFR) have low levels of PKA activity, and forskolin treatment failed to activate proenkephalin transcription (data not shown) or CREB phosphorylation (Figure 3B). However, FGF stimulated CREB phosphorylation at Ser133 (Figure 3B) in L6-FGFR myoblasts, suggesting a PKA-independent pathway. We also analyzed the phosphorylation of CREB at Ser133 *in vitro* using whole-cell extracts prepared from SK-N-MC cells treated with forskolin or FGF. A peptide inhibitor (PKI), which inhibits PKA selectively, greatly reduced control and forskolin-stimulated CREB kinase activity in cell extracts, but did not inhibit FGF-stimulated CREB Ser133-kinase activity significantly (Figure 3C). Taken together, the data suggest that FGF stimulates CREB's

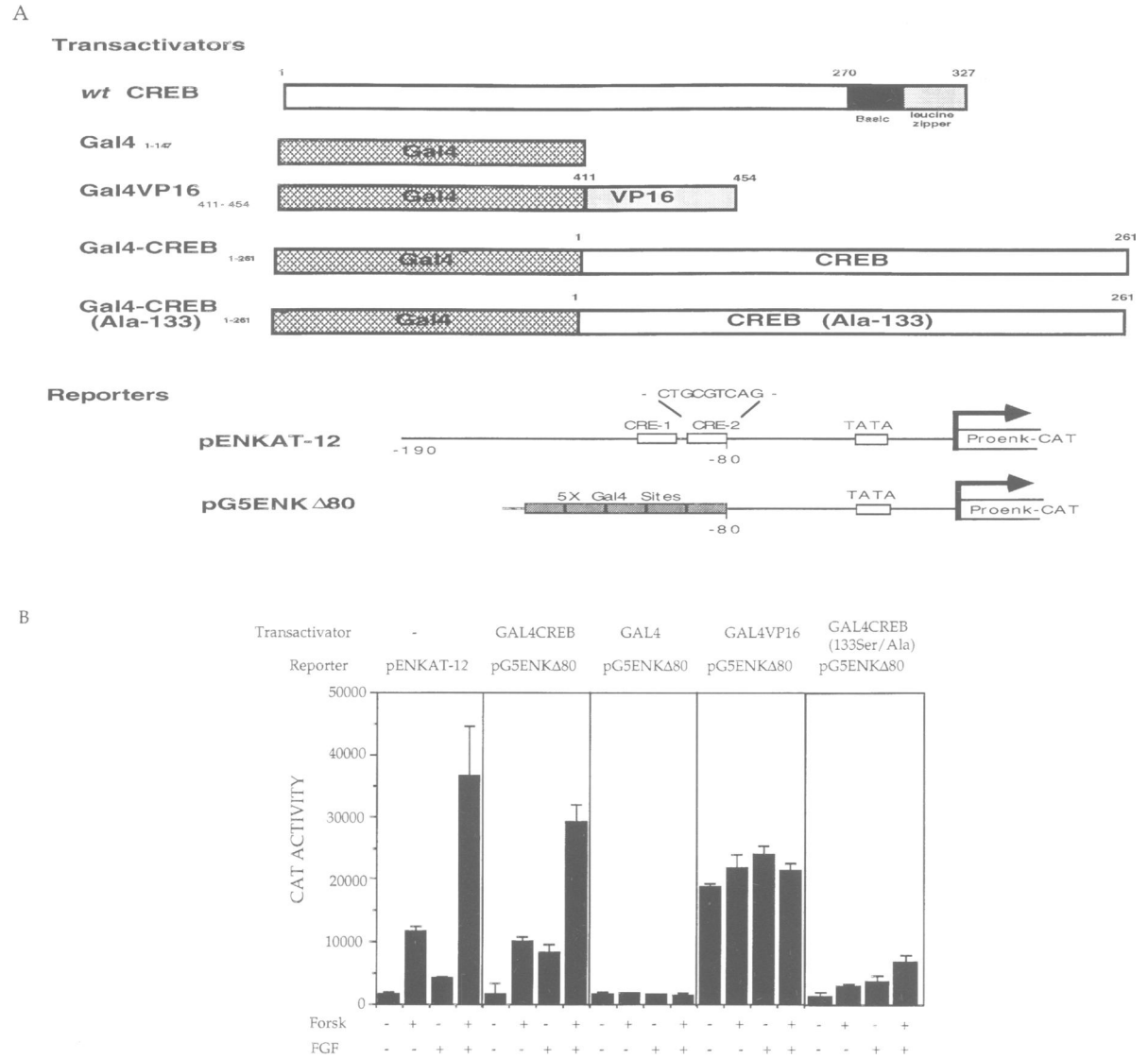


**Fig. 1.** CREB activates transcription in response to FGF. (**A**) Structure of the reporter and transactivator constructs. Expression of the indicated activators and activator fusion proteins is from the same RSV expression vector, pRSV424. Amino acid residues derived from GAL4, VP16 and CREB are indicated. Gal4–CREB (Ala-133) refers to an altered version of CREB in which Ser133 is changed to Ala (Lee *et al.*, 1990). The reporter gene, pENKAT-12, directing CAT expression from the human proenkephalin promoter has been described (Comb *et al.*, 1986) and the effects of FGF and cAMP previously mapped to the CRE-2 element (Comb *et al.*, 1988; Tan *et al.*, 1994). (**B**) FGF stimulates GAL4–CREB-dependent transcription and the transcriptional activation requires Ser133 of CREB. SK-N-MC cells were transfected with either 2.5 μg of pENKAT-12 or 10 μg of pG5ENK-Δ80, plus either 10 μg of pRSVGAL4–CREB, pRSVGAL4–VP16, pRSVGAL4 or GAL4–CREB containing a Ser133 to Ala mutation (133Ser/Ala) as indicated. 2 μg of pRSVLuciferase was co-transfected as an internal control to normalize transfection efficiency. At 18 h post-transfection, cells were treated with 10 μM forskolin or 25 ng/ml of basic FGF as indicated for 6 h. The mean CAT activity (± SEM) for three independent experiments using duplicate plates are indicated.

transcriptional activity via a pathway distinct from that activated by cAMP.

The binding of FGF to its receptor is known to induce receptor dimerization, autophosphorylation at Tyr766 and the activation of phospholipase C, which in turn activates protein kinase C (PKC). As Ser133 of CREB is contained within a consensus sequence for PKC phosphorylation and is phosphorylated by PKC *in vitro* (Yamamoto *et al.*, 1988), we next asked whether activation of phospholipase C is required for FGF-induced phosphorylation of Ser133 by analyzing whether an FGF receptor in which Tyr766 is mutated to Phe (Mohammadi *et al.*, 1992; Peters *et al.*, 1992), was capable of activating CREB phosphorylation in L6-FGFR cells. This mutation blocks FGF-induced activation of phospholipase C (Mohammadi *et al.*, 1992; Peters *et al.*, 1992), but has no effect on the ability of FGF to stimulate CREB phosphorylation (Figure 3D), indicating that activation of phospholipase C is not required for CREB phosphorylation. In addition, treatment of SK-N-MC cells with 200 nM TPA, an activator of several isoforms of PKC, had no effect on CREB phosphorylation (data not shown), while down-regulation of PKC by treatment of SK-N-MC cells for 24 h with TPA had no effect on the ability of FGF to stimulate CREB phosphorylation (Figure 3E).

Growth factors also activate $p70^{S6K}$, a protein kinase that is activated by a Ras-independent pathway (Ming *et al.*, 1994), and which appears to be triggered by the

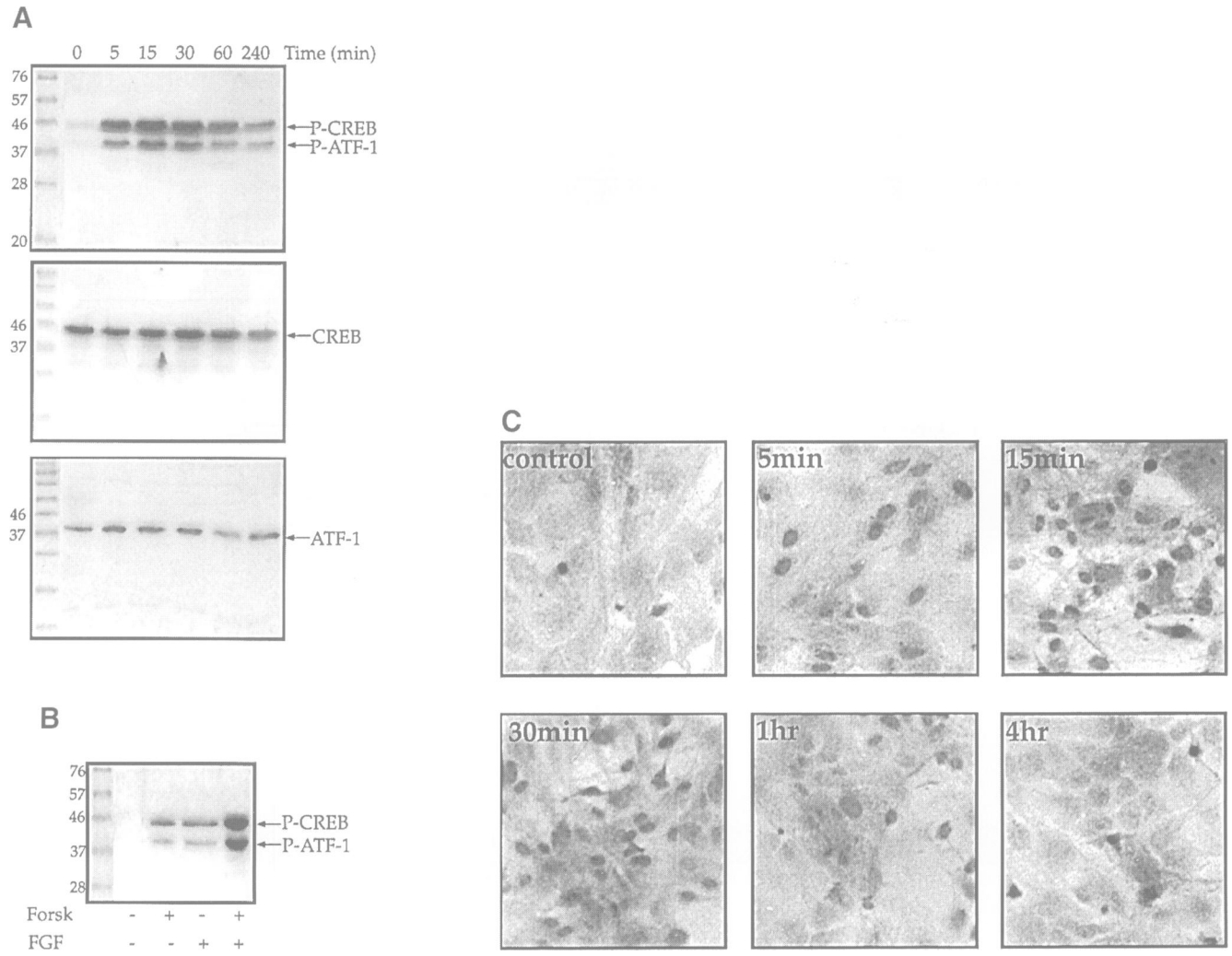


**Fig. 2.** FGF stimulates CREB phosphorylation at Ser133 in SK-N-MC cells and astrocytes. (**A**) FGF stimulates CREB phosphorylation at Ser133 in SK-N-MC cells. SK-N-MC cells were treated with 50 ng/ml basic FGF for the times indicated. Extracts were split and CREB phosphorylation analyzed by Western blotting using antibodies specific for phosphorylated Ser133 of CREB (P-CREB) or phosphorylated Ser63 of ATF-1 (P-ATF-1). CREB/ATF-1 expression was analyzed by Western blotting using antibodies that recognize either CREB or ATF-1 regardless of their phosphorylation status. Arrows indicate the position of CREB and ATF-1. (**B**) FGF and forskolin induce CREB phosphorylation in an additive fashion in SK-N-MC cells. SK-N-MC cells were treated with 10 μM forskolin or 25 ng/ml basic FGF for 15 min and CREB/ATF-1 phosphorylation was analyzed by Western blotting using antibodies which recognize CREB and ATF-1 phosphorylated at Ser133 and Ser63 respectively. (**C**) FGF induces nuclear phospho-CREB immunoreactivity in primary cultures of rat cerebral cortex type I astrocytes. Astrocytes isolated from rat cerebral cortex were maintained in culture for 1 week and treated with 25 ng/ml of FGF for the times indicated. Immunocytochemistry was performed with anti-phospho-CREB antibody.

activation of phosphatidylinositol 3-kinase (Chung *et al.*, 1994; Vlahos *et al.*, 1994). Moreover, the cAMP-responsive transcription factor CREM, which is related to CREB, was reported to be phosphorylated by $p70^{S6K}$ at the serine equivalent to Ser133 (de Groot *et al.*, 1994). However, the phosphorylation of CREB at Ser133 induced by FGF was unaffected by pretreatment of SK-N-MC cells with rapamycin (Figure 3F), a drug which inhibits the activation of $p70^{S6K}$ specifically (Chung *et al.*, 1992; Kuo *et al.*, 1992; Price *et al.*, 1992), whereas rapamycin completely blocked the activation of $p70^{S6K}$ by FGF in SK-N-MC cells (data not shown). Taken together, the data presented above demonstrate that FGF activation of CREB does not require PKA, phospholipase C, TPA-activated isoforms of PKC, or $p70^{S6K}$.

### *FGF-dependent CREB phosphorylation is inhibited by dominant negative Ras, but does not require the activation of $p90^{rsk}$ or SAPK/JNK*

Inhibition of Ras signaling by overexpression of a mutant Ras protein (H-Ras-Asn-17) (Feig and Cooper, 1988) blocked FGF-dependent gene activation, but had little or no effect on forskolin-stimulated gene expression (Figure 4A). As expected, inhibition of either PKA or Ras activity blocked the synergistic activation produced by FGF- and forskolin-activated signaling pathways (Figures 3A and 4A).

As FGF-dependent phosphorylation of CREB appeared to require Ras, we next sought to determine whether Ras-activated kinases might be involved. Ras is known to activate the kinase cascade comprising c-Raf, MAP kinase kinase, p42 and p44 MAP kinases, and $p90^{rsk}$. MAP

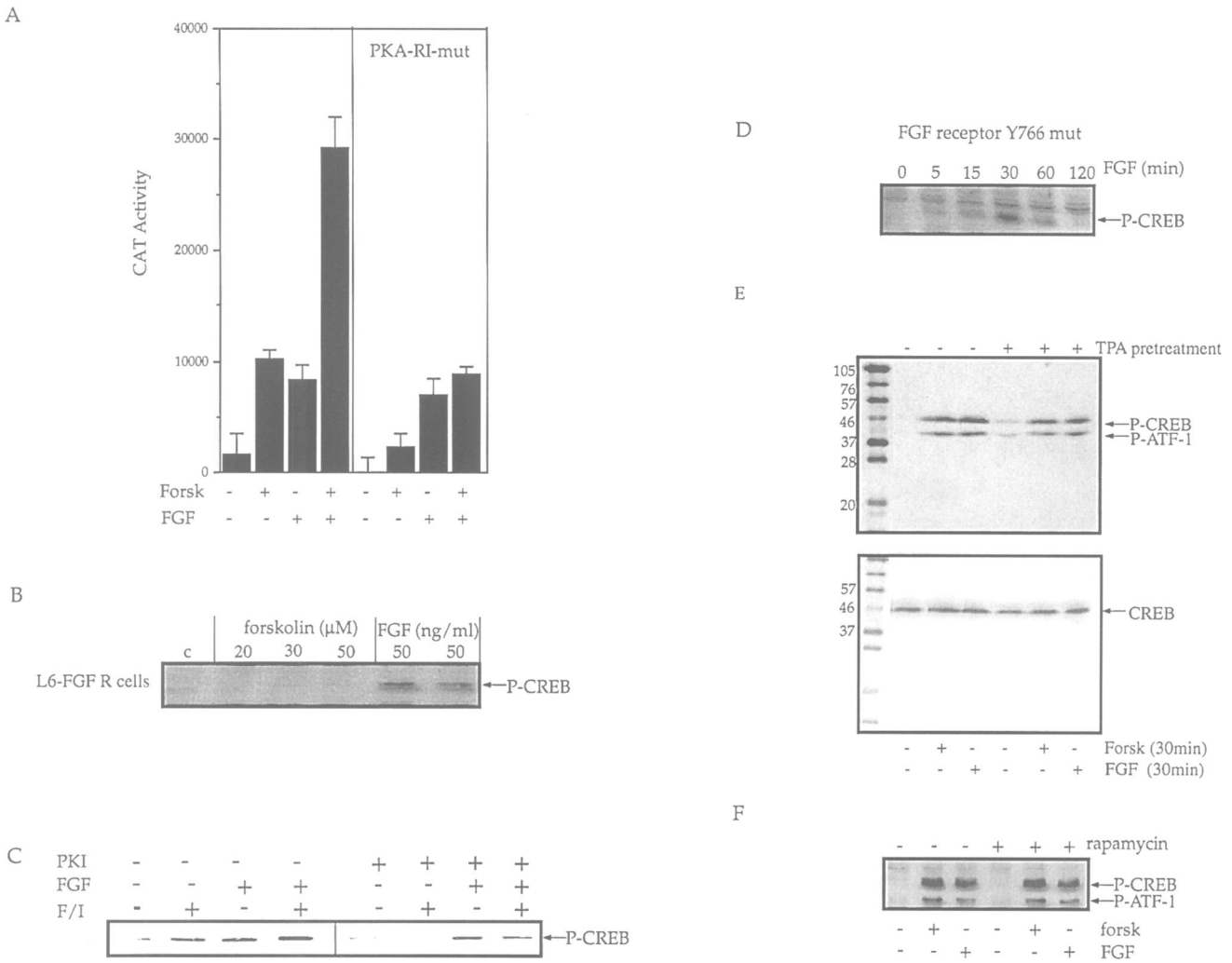


**Fig. 3.** Activation of CREB by FGF is independent of cyclic AMP-dependent protein kinase, protein kinase C, and p70 S6 kinase. (**A**) A dominant-inhibitory regulatory subunit of protein kinase A (PKA-RI-mut) blocks forskolin-, but not FGF-induced CREB activation. SK-N-MC cells were transfected with 10 μg of pG5ENK-Δ80 reporter, 10 μg of GAL4–CREB, 2 μg of pRSVLuciferase, and 20 μg of an expression vector expressing a mutant form of the regulatory type I subunit of the cyclic AMP-dependent protein kinase (PKA-RI-mut). At 18 h post-transfection, cells were treated for 6 h with 10 μM forskolin or 25 ng/ml of basic FGF as indicated. The mean CAT activity (± SEM) for three independent experiments using duplicate plates are indicated. (**B**) FGF, but not forskolin, stimulates CREB phosphorylation at Ser133 in L6-FGFR myoblasts. L6 myoblasts transfected with wild-type FGF receptor (Mohammadi *et al.*, 1992) were treated with 20, 30 and 50 μM forskolin or 50 ng/ml basic FGF for 30 min, and CREB phosphorylation analyzed by Western blotting using antibodies that recognize CREB phosphorylated at Ser133 (P-CREB). (**C**) Inhibition of protein kinase A in SK-N-MC cell extracts selectively inhibits forskolin-, but not FGF-induced kinase activity. Whole-cell extracts were prepared from SK-N-MC cells treated for 30 min with 10 μM forskolin plus 0.5 mM IBMX or 25 ng/ml of basic FGF as indicated. *In vitro* kinase assays were performed using 10 ng GST–CREB fusion protein (Tan *et al.*, 1994) as substrate (see Materials and methods). Phosphorylation of CREB at Ser133 was analyzed by Western blotting using phospho-CREB-specific antibodies. (**D**) Mutation of the FGF receptor at Tyr766 has no effect on FGF-induced CREB phosphorylation. L6 myoblasts transfected with an FGF receptor mutant in which Tyr766 is replaced by a Phe (Y766F) Myoblasts were treated with 50 ng/ml basic FGF for the times indicated and CREB phosphorylation was analyzed by Western blotting using phospho-CREB antibody (P-CREB). (**E**) Chronic treatment with TPA does not affect the ability of FGF to stimulate CREB phosphorylation. SK-N-MC cells were treated with 200 nM TPA for 24 h and then for 30 min with 10 μM forskolin or 25 ng/ml basic FGF. CREB phosphorylation and expression were analyzed by immunoblotting using antibodies which recognize CREB phosphorylated at Ser133 (P-CREB) or total CREB protein (CREB). (**F**) $p70^{S6K}$ does not mediate FGF-induced phosphorylation of CREB at Ser133. SK-N-MC cells were incubated for 60 min with rapamycin (40 nM), then stimulated for 15 min with FGF (50 ng/ml) and forskolin (25 μM). CREB phosphorylation was analyzed by Western blotting using phospho–CREB antibody.

kinases have a strict requirement for Ser/Thr-Pro sequences and are therefore incapable of phosphorylating CREB at Ser133. However, $p90^{rsk}$ is activated by MAP kinase and is known to phosphorylate proteins at Arg-Arg-Xaa-Ser motifs (Donella-Deana *et al.*, 1993; Sutherland *et al.*, 1993) which surround Ser133 of CREB, and has also been reported to phosphorylate CREB at Ser133 *in vitro* (Ginty *et al.*, 1994). We therefore tested whether $p90^{rsk}$ might be responsible for phosphorylating CREB at Ser133 in FGF-stimulated SK-N-MC cells. $p90^{rsk}$ was activated in response to FGF (Figure 4B) but activation was not blocked by SB 203580, a specific inhibitor of p38 MAP kinase, at doses that completely blocked the FGF-stimulated phosphorylation of CREB (Figure 4C). In addition, PD 98059, a specific inhibitor of MEK activation (Alessi *et al.*, 1995b), completely blocked FGF-induced MAPK and $p90^{rsk}$ activation but had little effect on CREB phosphorylation (data not shown).

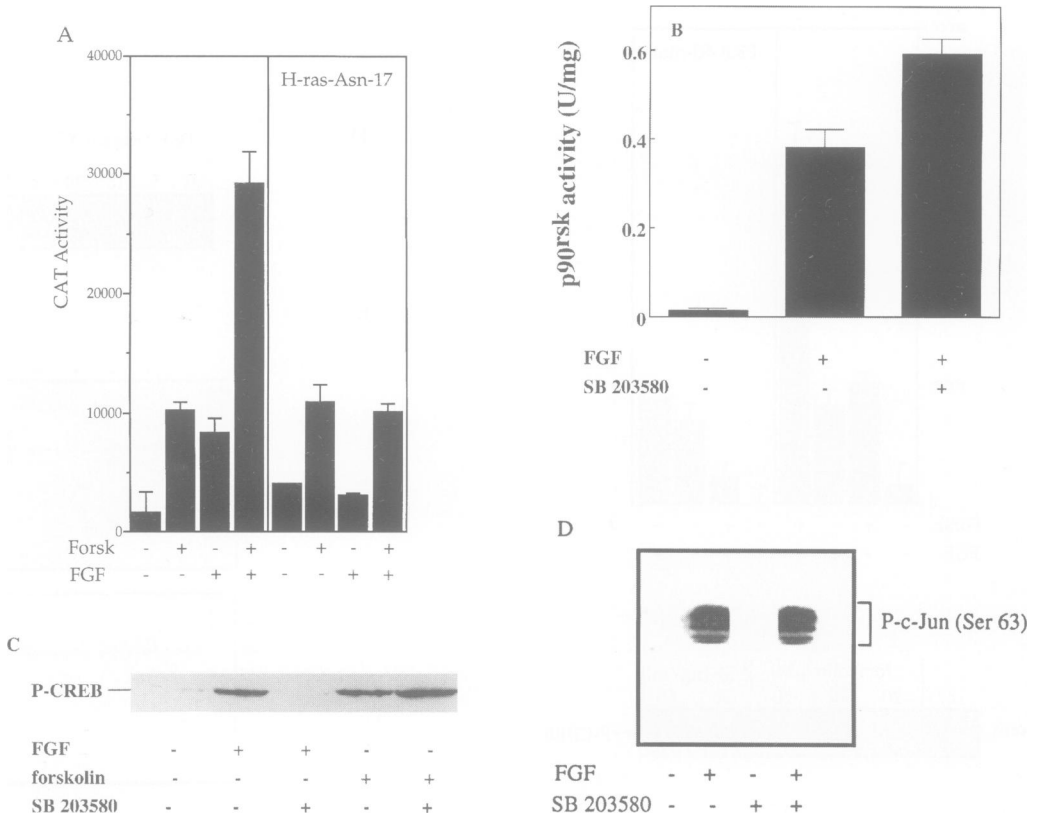


**Fig. 4.** Activation of CREB by FGF requires Ras and is prevented by SB 203580 but is not mediated by SAPK/JNK or $p90^{rsk}$. (**A**) A dominant-inhibitory-Ras blocks FGF-, but not forskolin-induced CREB activation. SK-N-MC cells were transfected with 10 μg of pG5ENK-Δ80 reporter, 10 μg of GAL4–CREB, 2 μg of pRSVLuciferase and 20 μg of an expression vector expressing a dominant-inhibitory-Ras protein (H-ras-Asn-17). At 18 h post-transfection, cells were treated for 6 h with 10 μM forskolin or 25 ng/ml of basic FGF as indicated. CAT activities were normalized to the level of pRSVLuciferase expression. The mean CAT activity (± SEM) for three independent experiments using duplicate plates are indicated. (**B**) SB 203580 has no effect on activation of $p90^{rsk}$ in SK-N-MC cells. SK-N-MC cells were incubated in the presence or absence of SB 203580 (10 μM) for 60 min and then stimulated with FGF (50 ng/ml) for a further 15 min in the continued presence of inhibitor. After cell lysis, $p90^{rsk}$ was immunoprecipitated and assayed as described in Materials and methods. The results are shown (± SEM) for three separate experiments. (**C**) SB 203580 prevents FGF- but not forskolin-induced, phosphorylation of CREB. SK-N-MC cells were incubated in the presence or absence of SB 203580 (10 μM) for 60 min and then stimulated with FGF (50 ng/ml) or forskolin (20 μM) for a further 15 min in the continued presence of inhibitor. CREB phosphorylation was analyzed by immunoblots using phospho-CREB antibody. (**D**) SB 203580 has no effect on activation of SAPK/JNK in SK-N-MC cells. SK-N-MC cells were incubated in the presence or absence of SB 203580 (40 μM) for 60 min and then stimulated with FGF (50 ng/ml) for a further 15 min in the continued presence of inhibitors. After cell lysis, JNK activity was measured using a GST–c-Jun pull-down assay by incubation with 10 μg of GST–c-Jun beads. c-Jun phosphorylation at Ser63 was assayed using a phospho-specific antibody against Ser63 of c-Jun, as described in Materials and methods.

We next examined whether FGF-induced stimulation of SAPK/JNK might result in activation of CREB phosphorylation. FGF induced SAPK activity but this activation was not blocked by SB 203580 at concentrations that completely blocked FGF-induced CREB phosphorylation (Figure 4D). These results indicate that $p90^{rsk}$ and SAPK/JNK are not the enzymes responsible for FGF-induced phosphorylation of CREB at Ser133.

### *The p38 MAP kinase/MAPKAP kinase-2 pathway mediates the activation of CREB by FGF or chemical stress*

The finding that SB 203580, a specific inhibitor of p38 MAP kinase (see Discussion), prevented FGF-induced CREB phosphorylation but not forskolin-stimulated CREB phosphorylation (Figure 4C), suggested the involvement of the p38 MAP kinase signaling pathway in mediating this effect of FGF. Moreover, the sequence surrounding Ser133 of CREB conforms to the minimum consensus (Leu-Xaa-Arg-Xaa-Xaa-Ser-Xaa-Xaa) required for phosphorylation by MAP kinase-activated protein kinase-2 (MAPKAP kinase-2) (Stokoe *et al.*, 1993), an immediate downstream target of p38 MAP kinase (Rouse *et al.*, 1994; Cuenda *et al.*, 1995), suggesting that MAPKAP kinase-2 might mediate the FGF-induced phosphorylation of CREB at Ser133. The following evidence demonstrated that this was indeed the case. First, we found that MAPKAP kinase-2 purified from rabbit skeletal muscle phosphorylated CREB at Ser133 *in vitro* (Figure 5A). Secondly, we found that the activation of MAPKAP kinase-2 (Figure 5C) occurred just prior to the phosphorylation of CREB (Figure 5B) in FGF-stimulated SK-N-MC cells. Thirdly, the FGF-induced activation of MAPKAP kinase-2 was prevented by SB 203580 at a concentration ($IC_{50}$ = 0.1 μM) similar to that which blocked the activation of CREB (Figure 6A). Fourthly, FGF-stimulation activated p38 MAP kinase in SK-N-MC cells (Figure 7A) and Gal4–CREB-dependent transcription at the proenkephalin promoter was stimulated by transfecting SK-N-MC cells with a plasmid expressing p38 MAP kinase, but not by transfection with a kinase-negative mutant of p38 MAP kinase or by transfection with p44 MAP kinase (Figure

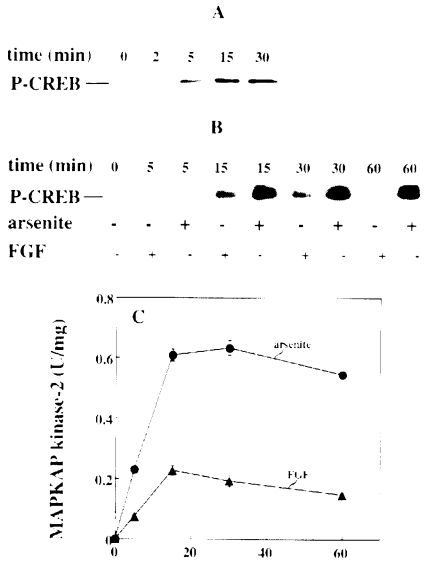


**Fig. 5.** MAPKAP kinase-2 activation and CREB phosphorylation in response to arsenite or FGF in SK-N-MC cells. (**A**) MAPKAP kinase-2 phosphorylates CREB on Ser133 *in vitro*. GST–CREB (5 ng/ml) was incubated at 30°C with rabbit skeletal muscle MAPKAP kinase-2 (10 U/ml) for the times indicated. The reactions were stopped by denaturation in SDS and after electrophoresis immunoblotting was carried out with antibodies that recognize the Ser133-phosphorylated form of CREB (P-CREB). (**B**) Cells were incubated for the times indicated with FGF (50 ng/ml) or sodium arsenite (0.5 mM). After cell lysis, aliquots of cell extract were denatured in 2% (by mass) SDS and analyzed by Western blotting using antibodies that recognize the Ser133-phosphorylated form of CREB. (**C**) Same as (B) except that, after cell lysis, MAPKAP kinase-2 was immunoprecipitated with the antibody raised against a C-terminal peptide of MAPKAP kinase-2 and assayed with MAPKAPtide.

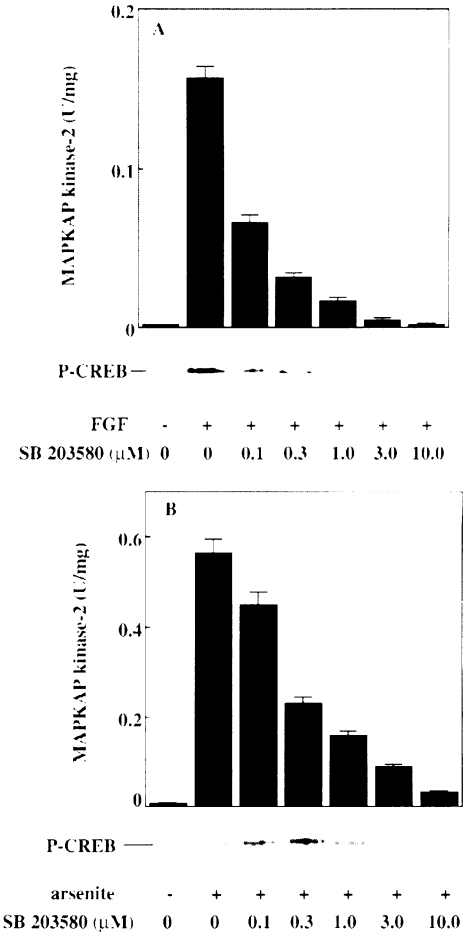


**Fig. 6.** Similar concentrations of SB 203580 prevent MAPKAP kinase-2 activation and CREB phosphorylation in FGF- or arsenite-stimulated SK-N-MC cells. (**A**) SK-N-MC cells were incubated for 60 min with the indicated concentrations of SB 203580 and then for a further 15 min with FGF (50 ng/ml) in the continued presence of inhibitor. After cell lysis, MAPKAP kinase-2 was immunoprecipitated with the C-terminal antibody and assayed. The results (± SEM) are shown for three separate experiments. Further aliquots of extract were denatured in 2% (by mass) SDS and Western blotting with antibodies that recognize Ser133-phosphorylated CREB. (**B**) Same as (A) except that cells were stimulated with sodium arsenite (0.5 mM) for 15 min instead of FGF.

8A). In the last-mentioned experiments, p38 MAP kinase activated Gal4–CREB 10-fold and transcription was activated a further 1.5-fold in the presence of FGF. p38 MAP kinase activity after transfection into SK-N-MC cells also correlated with its ability to activate Gal4–CREB (Figure 8B). In addition, p38 MAP kinase activation of CREB required Ser133, and Gal4–CREB was activated specifically, as Gal4–ATF-3 was not induced by p38 MAP kinase (data not shown) and Gal4–VP-16 was induced only modestly by p38 MAP kinase (1.3-fold) and FGF (1.6-fold) (Figure 8A).

The p38 MAP kinase/MAPKAP kinase-2 pathway is present in all mammalian cells examined so far, where it is activated by cytokines and cellular stresses, such as treatment with sodium arsenite. This chemical stress is known to mimic many of the effects of heat shock on gene expression and protein phosphorylation (Tanguay *et al.*, 1983; Welch, 1985). Arsenite was a stronger inducer of p38 MAP kinase activity (Figure 7B), MAPKAP kinase-2 activity (Figure 5C) and CREB phosphorylation at Ser133 (Figure 5B) than FGF. The arsenite-induced activation of MAPKAP kinase-2 was also prevented by SB 203580 at a concentration ($IC_{50}$ = 0.3 μM) slightly higher than that which prevented activation by FGF (Figure 6B). The requirement for slightly higher concentrations of SB 203580 to suppress arsenite-induced CREB phosphorylation is presumably a reflection of the greater MAPKAP kinase-2 activity induced by this agonist. Like the effect of FGF, the arsenite-induced activation of MAPKAP kinase-2 (Figure 5C) and phosphorylation of CREB (Figure 5B) was maximal at 30 min.

In order to investigate whether MAPKAP kinase-2 was the only SB 203580-sensitive CREB kinase in SK-N-MC cells, we stimulated cells with FGF or arsenite, chromatographed the lysates on Mono Q and assayed the fractions for their ability to phosphorylate the standard MAPKAP kinase-2 substrate peptide KKLNRTLSVA (termed here MAPKAPtide) and a synthetic peptide corresponding to residues 122–136 of CREB, termed here CREBtide. Fractions 1–5 (Figure 9A–F) represent Mono Q flow-through activity and showed no detectable SB-sensitive CREBtide-kinase activity. A single peak of FGF- or arsenite-inducible, SB 203580-sensitive kinase activity was detected with either substrate (Figure 9A–D) which eluted from Mono Q at 0.1 M NaCl. The following evidence demonstrated that this peak was MAPKAP kinase-2 or a closely related homolog. First, the activity ratio MAPKAPtide kinase:CREBtide kinase (5:1) was

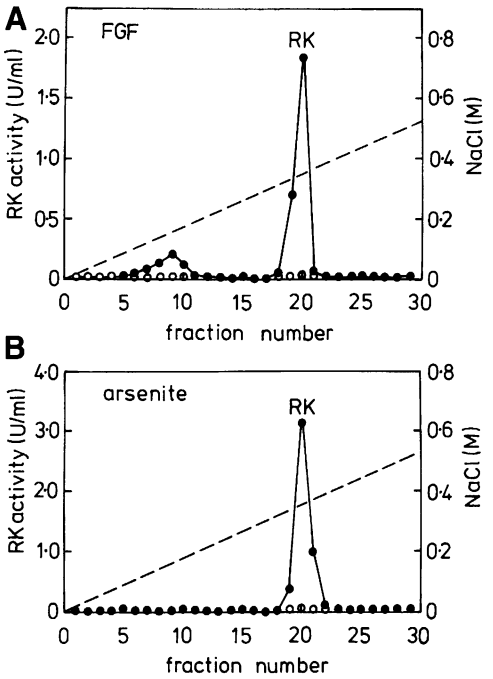


**Fig. 7.** Activation of p38 MAP kinase by FGF or arsenite in SK-N-MC cells. (**A**) SK-N-MC cells were incubated for 15 min with FGF (50 ng/ml), the cells lysed, and aliquots of extract (~0.4 mg protein) chromatographed on Mono Q. Fractions of 0.1 ml were assayed for p38 MAP kinase activity (●) as described in Materials and methods; ○, separate experiment using lysates from unstimulated cells. (**B**) Same as (A) except that cells were stimulated for 15 min with sodium arsenite (0.5 mM) instead of FGF.

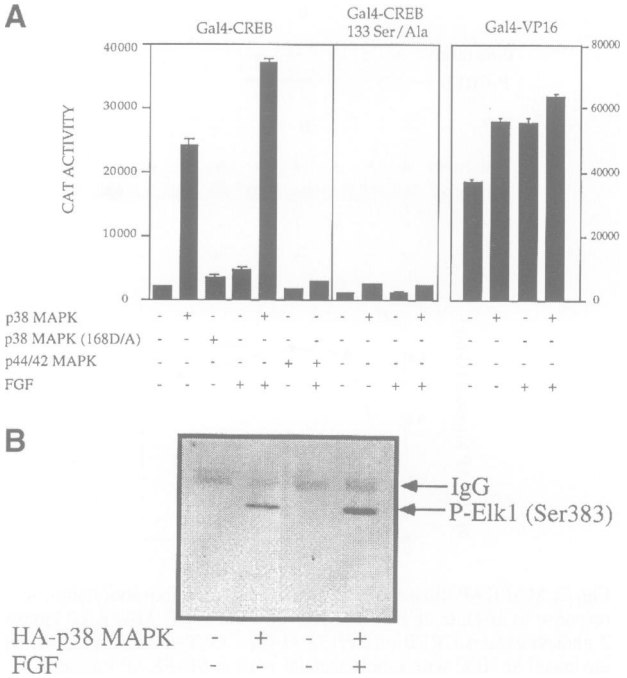


**Fig. 8.** p38 MAP kinase, but not p44 MAPK, activates Gal4–CREB at Ser133 in SK-N-MC cells. (**A**) SK-N-MC cells were transfected with 10 μg of pG5ENK-Δ80 as indicator, plus either 1.5 μg of pRSVGAL4–CREB, pRSVGAL4–CREB (Ser133→Ala), or pRSVGAL4–VP16, together with 5 μg pMT$_3$ p38 MAPK, 5 μg CMVp38/RK 168 D/A, or 5 μg CMVp44MAPK. At 18 h post-transfection, cells were treated with 25 ng/ml basic FGF for 6 h as indicated. The mean CAT activity (± SEM) for three independent experiments using duplicate plates are indicated. (**B**) Kinase activity of transfected p38 MAP kinase. SK-N-MC cells were transfected with HA-tagged p38 MAP kinase and cell extracts analyzed by immunoprecipitation using HA antibody. p38 MAP kinase activity was measured in an immunocomplex kinase assay using GST–Elk as a substrate. Phosphorylation of Elk-1 at Ser383 was analyzed by Western blotting using antibodies that selectively recognize Elk-1 phosphorylated at Ser383 (see Materials and methods).

identical to that of pure preparations of MAPKAP kinase-2 isolated from rabbit skeletal muscle (data not shown). Secondly, when the fractions were immunoprecipitated with an antibody raised against a C-terminal peptide from MAPKAP kinase-2 and the immunocomplexes were assayed for kinase activity, a single peak of activity was detected with either MAPKAPtide or CREBtide (Figure 9E and F) which coincided with the SB 203580-sensitive peak (Figure 9A–D). Moreover, immunoprecipitation using this antibody was quantitative with either substrate (compare Figure 9A–D with 9E and F) and immunoprecipitation was prevented if the antibody was first preincubated with the peptide immunogen (data not shown). These results were confirmed for both FGF- and arsenite-stimulated cells using a second antibody raised against three different peptides from MAPKAP kinase-2 (see Materials and methods). This antibody, which inactivates MAPKAP kinase-2 activity upon binding, immunodepleted 90% of the MAPKAPtide kinase and CREBtide kinase activity associated with the SB 203580-sensitive peak and immunodepletion did not occur if the antibody was first incubated with the peptide immunogens (data not shown). Results similar to those described above were also obtained when SK-N-MC cell extracts were analyzed using Mono S chromatography as described previously (Rouse *et al.*, 1994). Immunoblotting experiments using MAPKAP kinase-2-specific antisera detected the presence of 45 and 55 kDa proteins whose presence correlated with the peak of MAPKAP kinase-2 activity from Mono S (data not shown).

## Discussion

In this paper we identify and characterize an FGF- and arsenite-activated signaling pathway that regulates gene expression at a CRE in SK-N-MC cells. Basic FGF and cAMP stimulate proenkephalin gene expression synergistically in the human neuroblastoma cell line, SK-N-MC, and our work indicates that this occurs via a convergence of FGF- and cAMP-activated signaling pathways upon the transcription factor CREB at Ser133 to regulate its transcriptional activity. As FGF and arsenite also stimulate ATF-1 phosphorylation, and ATF-1 and CREB are known to form heterodimers, further analysis is required to determine whether FGF- and stress-induced gene expression is mediated by CREB and ATF-1 homo- or heterodimers. The FGF and stress-activated pathway is clearly distinct from the cAMP pathway and does not require phospholipase C-γ, TPA-activated isoforms of PKC, or the rapamycin-sensitive p70$^{S6K}$ pathway. The effects of FGF on CREB-dependent transcription at the proenkephalin CRE are inhibited by a dominant negative Ras mutant, but are not mediated by the Ras-dependent pathway which activates p42 and p44 MAP kinases, because the drug PD 98059 has little effect on FGF-stimulated CREB phosphorylation even though it abolishes

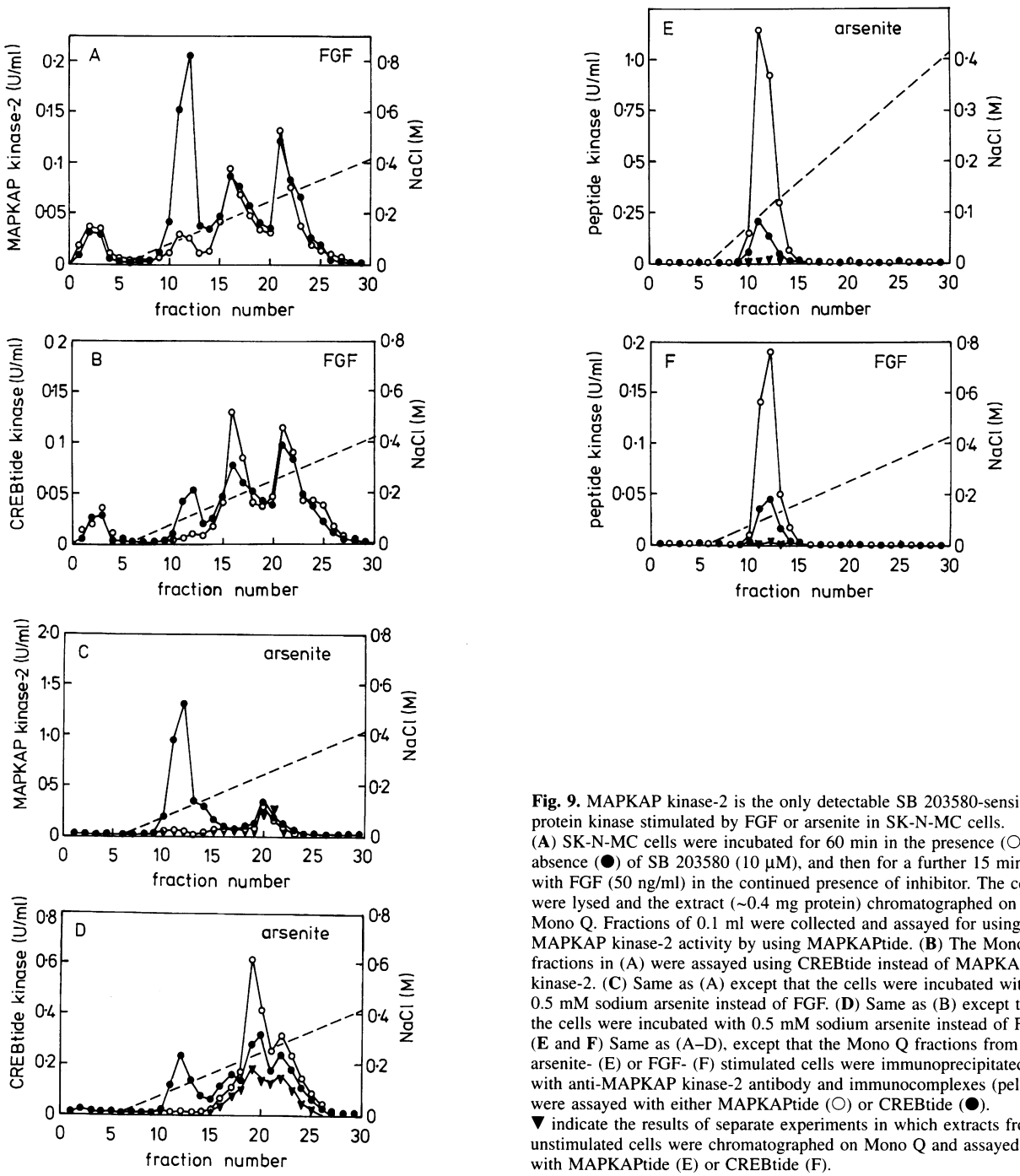


**Fig. 9.** MAPKAP kinase-2 is the only detectable SB 203580-sensitive protein kinase stimulated by FGF or arsenite in SK-N-MC cells. (**A**) SK-N-MC cells were incubated for 60 min in the presence (○) or absence (●) of SB 203580 (10 μM), and then for a further 15 min with FGF (50 ng/ml) in the continued presence of inhibitor. The cells were lysed and the extract (~0.4 mg protein) chromatographed on Mono Q. Fractions of 0.1 ml were collected and assayed for using MAPKAP kinase-2 activity by using MAPKAPtide. (**B**) The Mono Q fractions in (A) were assayed using CREBtide instead of MAPKAP kinase-2. (**C**) Same as (A) except that the cells were incubated with 0.5 mM sodium arsenite instead of FGF. (**D**) Same as (B) except that the cells were incubated with 0.5 mM sodium arsenite instead of FGF. (**E** and **F**) Same as (A–D), except that the Mono Q fractions from arsenite- (E) or FGF- (F) stimulated cells were immunoprecipitated with anti-MAPKAP kinase-2 antibody and immunocomplexes (pellets) were assayed with either MAPKAPtide (○) or CREBtide (●). ▼ indicate the results of separate experiments in which extracts from unstimulated cells were chromatographed on Mono Q and assayed with MAPKAPtide (E) or CREBtide (F).

activation of $p90^{rsk}$ which lies immediately 'downstream' of p42 and p44 MAP kinases (data not shown). Conversely, the drug SB 203580 completely blocks the activation of CREB without affecting the activation of $p90^{rsk}$ (Figures 4B and C).

CREB and ATF-1 are also known to become phosphorylated at Ser133 and Ser63 respectively in response to signals that elevate the intracellular concentration of calcium ions (Liu *et al.*, 1993; Ghosh and Greenberg, 1995). In addition, calmodulin-dependent protein kinase II and calmodulin-dependent protein kinase IV have been shown to phosphorylate CREB at this site *in vitro* (Sheng *et al.*, 1991). However, the mechanism by which FGF or arsenite stimulates CREB phosphorylation at Ser133 cannot involve elevations of intracellular $Ca^{2+}$ followed by activation of $Ca^{2+}$/calmodulin-dependent protein kinases, because the activities of $Ca^{2+}$/calmodulin-dependent kinases II and IV are unaffected by SB 203580, even at 100 μM (J.Rouse, unpublished results).

Our results indicate that FGF and arsenite stimulate CREB phosphorylation at Ser133 via a pathway that involves p38 MAP kinase and MAPKAP kinase-2. In this pathway, the nuclear transcription factor CREB is phosphorylated by MAPKAP kinase-2, although we cannot rule out the involvement of other closely related MAPKAP kinase-2 homologs that may also react with the two antibodies used in this study. Immunoblotting studies suggest that two different MAPKAP kinase-2 immuno-

reactive species of ~55 kDa and ~45 kDa are present in SK-N-MC cells and co-purify with MAPKAP kinase-2 activity, raising the possibility that either or both proteins may be involved in CREB activation. MAPKAP kinase-2 contains a bipartite nuclear localization sequence at its C-terminus (Stokoe *et al.*, 1993) and is present in the nuclei of mammalian cells (R.Ben Levy and C.J.Marshall, personal communication). Although MAPKAP kinase-2 can be activated by p42 and p44 MAP kinases *in vitro* (Stokoe *et al.*, 1992), it is activated *in vivo* by the p38 MAP kinase, because activation is prevented by SB 203580 (Ben Levy *et al.*, 1995; Cuenda *et al.*, 1995), a compound which inhibits p38 MAP kinase, but not p42 or p44 MAP kinases (Cuenda *et al.*, 1995) or the activation of $p90^{rsk}$ (Figure 4). The activation of MAPKAP kinase-2 by arsenite in KB cells is accompanied by its phosphorylation at six residues, namely Ser9, Thr25, Thr222, Ser272, Thr334 and Thr338 (Ben Levy *et al.*, 1995). Of these residues, Thr25, Thr222, Ser272 and Thr334 are phosphorylated by p38 MAP kinase, while phosphorylation of Thr338 appears to be catalyzed by MAPKAP kinase-2 itself, after it has been activated. The activation of MAPKAP kinase-2 is unusual, being triggered by the phosphorylation of any two of the three residues Thr222, Ser272 and Thr334 (Ben Levy *et al.*, 1995), but the reason for this complexity is not yet known.

The identity of the protein kinase(s) which activates p38 MAP kinase *in vivo* is also still uncertain. Three MAP kinase kinase homologs have been cloned, termed MKK3 (Derijard *et al.*, 1995; Raingeaud *et al.*, 1995), MKK4/SEK-1 (Sanchez *et al.*, 1994; Derijard *et al.*, 1995) and MKK6 (Cuenda *et al.*, 1996; Han *et al.*, 1996; Raingeaud *et al.*, 1996) that are capable of activating p38 MAP kinase *in vitro* or after co-transfection. While MKK4/SEK-1 activates both SAPK/JNK and p38 MAP kinase (Derijard *et al.*, 1995), MKK3 and MKK6 appear to activate p38 MAP kinase selectively (Raingeaud *et al.*, 1996), and MKK6 (also termed SAPKK-3) is the major p38 MAPK activator induced by cellular stresses or interleukin-1 in human epithelial KB cells, in bacterial lipopolysaccharide-stimulated THP.1 human monocytes (Cuenda *et al.*, 1996; Meier *et al.*, 1996) or in rabbit skeletal muscle. Overexpression of constitutively active forms of MKK3 and MKK6 have been shown to stimulate p38 MAP kinase activity, to induce phosphorylation of ATF-2 and Elk-1 and to activate ATF-2- and Elk-1-mediated gene transcription (Raingeaud *et al.*, 1996). The role of these three different MAP kinase kinase homologs in FGF- and stress-induced CREB/ATF-1 activation requires further investigation.

p42/44 MAP kinase, SAPK/JNK and p38 MAP kinase all appear to directly phosphorylate and activate the transcription factors Elk-1 and AFT-2 *in vitro* and in transfected cells (Marais *et al.*, 1993; Cavigelli *et al.*, 1995; Raingeaud *et al.*, 1996). However, unlike the direct phosphorylation of Ser-Pro residues within Elk-1 and ATF-2, the phosphorylation of CREB is not catalyzed by p38 MAP kinase directly, but by the intervening kinase MAPKAP kinase-2 or a closely related homolog. This serves to switch phosphorylation from Ser-Pro-directed targets, such as Elk-1 and ATF-2, to the PKA/calmodulin-dependent protein kinase specificity found at Ser133, resulting in the activation of CREB/ATF-1 and CRE-driven target genes. Activation of MAPKAP kinase-2 via its multiple activation sites may not only serve to further amplify and focus gene activation towards CRE-like targets, but may also serve to integrate signals from other pathways.

Our finding that SB 203580 prevents the FGF-induced phosphorylation of CREB, but not phosphorylation induced by forskolin (Figure 4C) reinforces the remarkable specificity of this drug that has been emerging from recent studies. Thus, SB 203580 blocks the TNF-induced production of interleukin-6 and the transcription of a CAT reporter containing two upstream NFκB binding sites in L929 and HeLa cells, but does not block the TNF-induced phosphorylation of the NFκB subunits (Rel-A and Rel-B), their dissociation from IκB or their ability bind to DNA; nor does it inhibit TNF-induced cytotoxicity in these cells (Beyaert *et al.*, 1996). Similarly, SB 203580 prevents the induction of c-Fos and c-Jun by cellular stresses in C3H 10t1/2 fibroblasts, but not their induction by EGF or phorbol esters (Hazzalin *et al.*, 1996). SB 203580 prevents the stimulation of glucose uptake by interleukin-1 in KB cells, but not the stimulation of glucose uptake by insulin-like growth factor-1 (Gould *et al.*, 1995). SB 203580 inhibits p38 MAP kinase at submicromolar concentrations but, even at 100 μM, has no effect on the activity of ~20 other protein kinases tested (including other MAP kinase homologs), and does not prevent the activation of any other growth factor or stress-activated signaling pathways examined (Lee *et al.*, 1994a; Cuenda *et al.*, 1995).

It has been reported that NGF stimulates CREB phosphorylation in PC12 cells by activating a 105 kDa protein kinase that elutes from Mono Q at 180 mM NaCl (Ginty *et al.*, 1994). This enzyme cannot be MAPKAP kinase-2, which is a monomeric 45 kDa enzyme (Stokoe *et al.*, 1992; Ben Levy *et al.*, 1995) that is not activated by NGF in PC12 cells (Rouse *et al.*, 1994; Cuenda *et al.*, 1995) and which in SK-N-MC cells elutes from Mono Q at 0.1 M NaCl (Figure 9). Moreover, we have found that, in PC12 cells, SB 203580 blocks the arsenite-induced phosphorylation of CREB, but not the NGF-stimulated phosphorylation of CREB (J.Rouse, unpublished data). The identity of the NGF-stimulated CREB kinase in PC12 cells remains to be established and suggests the existence of other growth factor-stimulated pathways leading to CREB activation.

Finally, activation of proenkephalin transcription by FGF- and stress-induced signaling pathways may play an important role in opioid-mediated stress responses. Activation of opioid systems involves both the release of opioid peptides as well as the induction of opioid gene expression. In general, these actions serve to restore cellular homeostasis by dampening the effects of over-activating stress and pain pathways (Lightman and Young, 1987; Borsook *et al.*, 1994). In addition to their abundant CNS expression, opioids are expressed in astrocytes and immune cells where their synthesis is stimulated by IL-1 and TNFα (Low *et al.*, 1992; Linner *et al.*, 1993), all of which activate p38 MAP kinase in other cells (Freshney *et al.*, 1994; Cuenda *et al.*, 1995). It will therefore be important to examine the role of the p38 MAP kinase pathway in the regulation of CREB phosphorylation and opioid gene expression in both immune and neural cells.

Of particular interest will be the role of p38 MAP kinase and MAPKAP kinase-2 in the hypothalamus and hippocampus, where dynamic changes in CREB phosphorylation and opioid expression are important for long-term changes in neural plasticity including stress-induced adaptations (Lightman, 1988), the maintenance of LTP/LDP (Morris and Johnston, 1995) and long-term memory (Bourtchuladze *et al.*, 1994; Tully *et al.*, 1994).

## Materials and methods

### Materials

Forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), chlorophenylthioadenosine 3′5′-cyclic monophosphate (cpt-cAMP), *N*-butyryl coenzyme A were from Sigma. Basic FGF, fetal calf sera (FCS) and mammalian cell culture media were from Gibco-BRL. Protein kinase A and PKI were from New England Biolabs (NEB). [$^{32}$P]dCTP and [$^{3}$H]chloramphenicol were from NEN Research Products. Phospho-CREB (Ser133) antibodies were from NEB and UBI, while CREB antibody was from NEB. Phospho-c-Jun (Ser63) and phospho-Elk-1 (Ser383) antibodies were from NEB. ATF-1 antibodies were from Santa Cruz Biotechnology. SB 203580 was kindly provided by J.Lee and P.Young (SmithKline Beecham, King of Prussia, PA), PD 98059 by A.Saltiel (Parke-Davis, Ann Arbor, MI), and rapamycin by S.Schreiber (Department of Chemistry, Harvard University). GST–c-Jun (1–141) was obtained from J.Woodgett. GST–Elk (307–428) was obtained from R.Treisman.

### Cell culture

SK-N-MC neuroblastoma cells and L6 myoblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS and supplemented with 2 mM glutamine. Primary cultures of rat cortical type I astrocytes were isolated and cultured from postnatal day 1–2 rat pups in DMEM/F12 with 10% (v/v) FCS as described previously (Melner *et al.*, 1990).

### Plasmid and DNA constructions

The following proenkephalin promoter–CAT constructs have been described previously: pENKAT-12 (Comb *et al.*, 1986), pRSVLuc (Maxwell *et al.*, 1989), GAL4 (Sadowski and Ptashne, 1989), GAL4–VP16 (Sadowski and Ptashne, 1989), GAL4–CREB (Lee *et al.*, 1990), Gal4–CREB (Ala133) (Lee *et al.*, 1990), Ha-Ras (Asn-17) (Feig and Cooper, 1988), PKA–RI-mut (Clegg *et al.*, 1987) and GST–Elk (Marais *et al.*, 1993). pRSV424 (Tan *et al.*, 1994) was constructed from pSV424 (Sadowski and Ptashne, 1989) by replacing the SV40 sequences with the RSV promoter/enhancer. pG5ENK-Δ80 was constructed from pENKAT-12 by replacement of proenkephalin promoter sequences from −195 to −80 with five copies of the Gal4 DNA binding site (Tan *et al.*, 1994). pCMVp38/RK was obtained from A.Zervos and pCMVp44MAPK was obtained from J.Avruch and GST-c-Jun (1–141) was from J.Woodgett. pCMVp38/RK 168 D/A was obtained from P.Young.

### Antibody production

Phospho-CREB (Ser133) antibody (NEB) detects CREB only when activated by phosphorylation at Ser133, and does not cross-react with up to 1 μg of bacterially expressed non-phosphorylated CREB. This antibody was produced by immunizing rabbits with a synthetic phospho-Ser133 peptide (KLH-coupled) corresponding to residues 129–137 (SRRPRS*YRKI) of human CREB. This antibody also detects phosphorylated Ser63 of ATF-1 which shares the sequence RRPRS*YRKI with CREB. Some experiments used a phospho-CREB antibody obtained from UBI. CREB antibody (NEB) was produced by immunizing rabbits with a synthetic peptide corresponding to residues 123–137 (KRREILSRRPSYRK) of human CREB. This antibody is CREB-specific and does not detect ATF-1. Phospho-c-Jun (Ser63) antibody (NEB) detects c-Jun only when activated by phosphorylation at Ser63. This antibody was produced by immunizing rabbits with a synthetic phospho-Ser63 peptide (KLH-coupled) corresponding to residues 59–67 (DLLTS*PDVGC) of human c-Jun. This antibody is highly phospho-specific as it shows no cross-reactivity with up to 1 μg of bacterially expressed c-Jun (non-phosphorylated) and does not cross-react with either JunB or JunD. Phospho-Elk-1 antibody (Ser383) (NEB) detects Elk-1 protein only when phosphorylated at Ser383, and was produced by immunizing rabbits with a synthetic phospho-Ser383 peptide (KLH-coupled) corresponding to residues 379–392 (WSTL*SPIAPRSPAKC) of human Elk-1. No cross-reactivity is seen with up to 1 μg of bacterially expressed non-phosphorylated Elk-1. MAPKAP kinase-2 antibodies were raised in sheep against a peptide near the C-terminus of MAPKAP kinase-2 (Gould *et al.*, 1995) and against a mixture of three different peptides from MAPKAP kinase-2 (Cuenda *et al.*, 1995) and affinity-purified as described previously. Antibodies that immunoprecipitate p70$^{S6K}$ (Alessi *et al.*, 1995a) and both the p90$^{rsk-1}$ and p90$^{rsk-2}$ isoforms [also called MAPKAP kinase-1α and MAPKAP kinase-1β (Alessi *et al.*, 1995a)] were raised in sheep and affinity-purified on peptide–Affigel 15 columns. Antisera that immunoprecipitate p38 MAP kinase (Rouse *et al.*, 1994) were raised in rabbits and were generously provided by A.Nebreda (European Molecular Biology Laboratory, Heidelberg).

### Transient transfections and CAT assay

Transient transfection of SK-N-MC cells was performed as described previously (Chu *et al.*, 1994) with various amounts of expression plasmid and pRSVLuc as described in the figure legends. The total amount of DNA transfected was maintained at 35 μg with pGEM3. Following calcium phosphate transfections, cells were glycerol-shocked and incubated for 16 h in media containing 0.5% FCS. Cells were treated with forskolin (10 μM) or FGF (25 ng/ml) for 6 h. Cells were lysed and CAT (Comb *et al.*, 1988) or luciferase (Brasier *et al.*, 1989) activity was determined as previously described.

### Bacterial expression of GST fusion proteins

GST fusion proteins were expressed in *Escherichia coli*, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and purified with glutathione (GSH)–agarose beads as described previously (Tan *et al.*, 1994).

### Immunoblotting

Cells were grown in 6-well plates for 2 days in medium containing 0.5% FBS, and treated with 10 μM forskolin or 50 ng/ml FGF for the times indicated. Cell extracts were prepared by lysing cells in 100 μl of SDS sample buffer (2% SDS, 10% glycerol, 80 mM Tris, pH 6.8, 0.15 M 2-mercaptoethanol, 0.02% bromophenol blue). Extracts from $2\times10^5$ cells (20 μl) were fractionated by 12% SDS–PAGE and transferred to PVDF membranes. Blots were blocked in 5% non-fat dry milk for 2 h at room temperature, then incubated with primary antibody overnight at 4°C, and for 1 h at room temperature with goat anti-rabbit alkaline phosphatase secondary antibody, and visualized using the CDP–Star Western detection system (NEB).

### Immunocytochemistry

Primary cultures of rat cortical type I astrocytes were isolated from postnatal day 1 rat pups and grown in DMEM/F12 with 10% FBS for 1 week and in DMEM/F12 with 0.5% FBS for 2 days (Melner *et al.*, 1990). After treatment, cells were fixed in 4% paraformaldehyde for 30 min at room temperature and immunocytochemistry was performed as described previously (Ginty *et al.*, 1993).

### Chromatography of cell lysates on Mono Q

Each 6-cm dish of SK-N-MC cells was lysed in 0.2 ml of buffer A (20 mM Tris–acetate, pH 7,0, 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 1% Triton X-100, 10 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, 2 μM microcystin, 1 mM benzamidine, 4 μg/ml leupeptin and 0.1% 2-mercaptoethanol) and centrifuged for 5 min at 13 000 *g*. The supernatants were removed, immediately frozen in liquid nitrogen and stored at −80°C. Thawed lysate (400 μg protein) was diluted to 0.2 ml with 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 5% (v/v) glycerol, 0.03% (w/v) Brij 35, 1 mM benzamidine, 0.3 mM sodium orthovanadate, 0.1% 2-mercaptoethanol (buffer A) and applied to a 5×0.16 cm Mono Q column equilibrated in buffer A. After washing with 0.4 ml of buffer, the column was developed with a 4 ml linear salt gradient to 0.7 M NaCl in buffer A. The flow rate was 0.2 ml/min and fractions of 0.1 ml were collected and assayed for protein kinase activities.

### Immunoprecipitation of protein kinases

Aliquots (10 μl) of a 50% suspension of protein G–Sepharose equilibrated in cell lysis buffer were incubated with affinity-purified anti-peptide antibodies raised against MAPKAP kinase-2 (1 μg), p90$^{rsk}$ (1 μg) or p70$^{S6K}$ (5 μg), or p38 MAP kinase (3 μl). After shaking for 30 min at 4°C, the suspensions were centrifuged, the pellets washed three times in lysis buffer, and aliquots of cell lysate (~50 μg protein) added. After shaking for a further 90 min at 4°C, the pellets were collected by centrifugation, washed twice with lysis buffer containing 0.5 M NaCl

and three times with lysis buffer, and then assayed for the appropriate protein kinase activity. Since the anti-p38 MAP kinase antibodies were inhibitory, immunoprecipitation of this enzyme was assessed by measuring depletion of activity from the supernatants, rather than by its appearance in the immunoprecipitates.

### *Protein kinase assays*

MAPKAP kinase-2 activity was measured by the phosphorylation of MAPKAPtide (KKLNRTLSVA) as described previously (Stokoe *et al.*, 1993), and CREBtide kinase activity was measured in an identical manner except that 30 μM CREBtide (CKRREILSRRPSYRK), a synthetic peptide corresponding to residues 122–136 of CREB, replaced 30 μM KKLNRTLSVA. p38 MAP kinase activity was measured by its ability to activate a form of MAPKAP kinase-2 [GST–MAPKAP kinase-2 (46–400) (Ben Levy *et al.*, 1995)] using the assay described by Rouse *et al.* (1994). $p90^{rsk}$ was assayed using a 32-residue peptide related to the C-terminus of ribosomal protein S6 (Dent *et al.*, 1990) and $p70^{S6K}$ using the peptide KRRRLASLRA (Baxter *et al.*, 1995). The detailed protocols were carried out essentially as described in Stokoe *et al.* (1993) and, apart from p38 MAP kinase, one unit of protein kinase activity was that amount which catalyzed the incorporation of 1 nmol of phosphate into substrate in 1 min. One unit of p38 MAP kinase activity was that amount which increased the activity of MAPKAP kinase-2 by one unit in 1 min. SAPK/JNK1 kinase activity was measured by binding of JNK to the c-Jun transactivation domain (Derijard *et al.*, 1994). SK-N-MC cells were lysed in buffer A (see above) and then incubated with 10 μl of GST–c-Jun immobilized on GSH–agarose beads at 4°C overnight. Beads were washed three times with buffer A and twice with kinase buffer, and kinase assays performed at 30°C for 30 min using 10 μl of GST–c-Jun immobilized on GSH–agarose beads as a substrate. Reactions were stopped by adding Läemmli Sample buffer, followed by PAGE and immunoblotting using phospho-specific c-Jun antibody that recognizes c-Jun only when phosphorylated at Ser63. p38 MAP kinase assays were carried out as follows. SK-N-MC cells transfected with HA-pCMVp38 MAP kinase were lysed in buffer A and incubated with HA antibody overnight, followed by protein A–Sepharose beads for 2 h at 4°C. The beads were washed three times with buffer A, twice with kinase buffer, and kinase assays were performed using 1 μg GST–Elk-1 (307–428) as a substrate. Elk-1 phosphorylation was analyzed by immunoblotting using a phospho-specific Elk-1 antibody that recognizes Elk-1 only when phosphorylated at Ser383.

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